(12) UK Patent Application (19) GB (11) 2 214 518 (13) A

(43) Date of A publication 06.09.1989

(21) Application No 8801870.0

(22) Date of filing 28.01.1988

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(51) INT CL⁴
G01N 21/64 33/483

(52) UK CL (Edition J)
C6F FK
G1B BAA
U1S S1411 S1427 S2135 S2155

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(58) Field of search
UK CL (Edition J) C6F FK FL, G1B BAA BBV
INT CL⁴ G01N 21/64 33/483
ESA Online Search in: (i) Chem. Abs. (ii) Biosis
(iii) Current Bio. Ab. (iv) Pascal

(54) Process and equipment for the rapid determination of the spermium cell count and/or living spermium count

(57) The process comprises dissolving the fluorescent dyestuff propidium iodide in a buffer and measuring the intensity (F_0), then adding the sperm sample and, after measurement of the intensity (F_1) and addition of a cytoplasm membrane- permeabilizing agent e.g. Saponin, Dibitonin or Nystatin, determining the intensity (F_2), then adding a buffer and a permeabilizing agent to the buffer and measuring the intensity (F_3) and subsequently the intensity (F_4) of the pure buffer and finally the intensity (F_5) of the sperm mixture added to the buffer, and calculating the cell concentration by using the formula:

$$\text{cell concentration} = \alpha \frac{(F_2 - F_3) - (F_5 - F_4)}{F_0} \text{ million/mm}^3$$

wherein α is the multiplication product from the slope of the calibration curve with the predilution ratio; and calculating the living cell ratio by using the formula:

$$\text{living cell \%} = 100 - \frac{(F_1 - F_0) - (F_5 - F_4)}{(F_2 - F_3) - (F_5 - F_4)} \cdot 100.$$

At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

GB 2 214 518 A

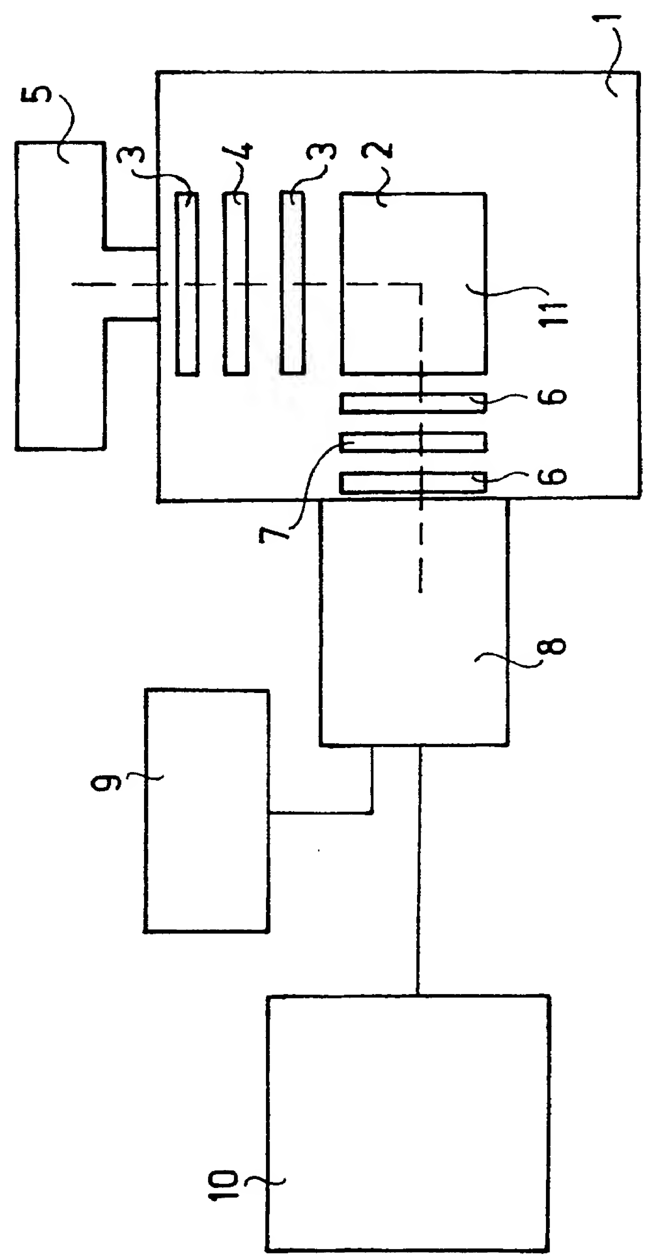


Fig.1

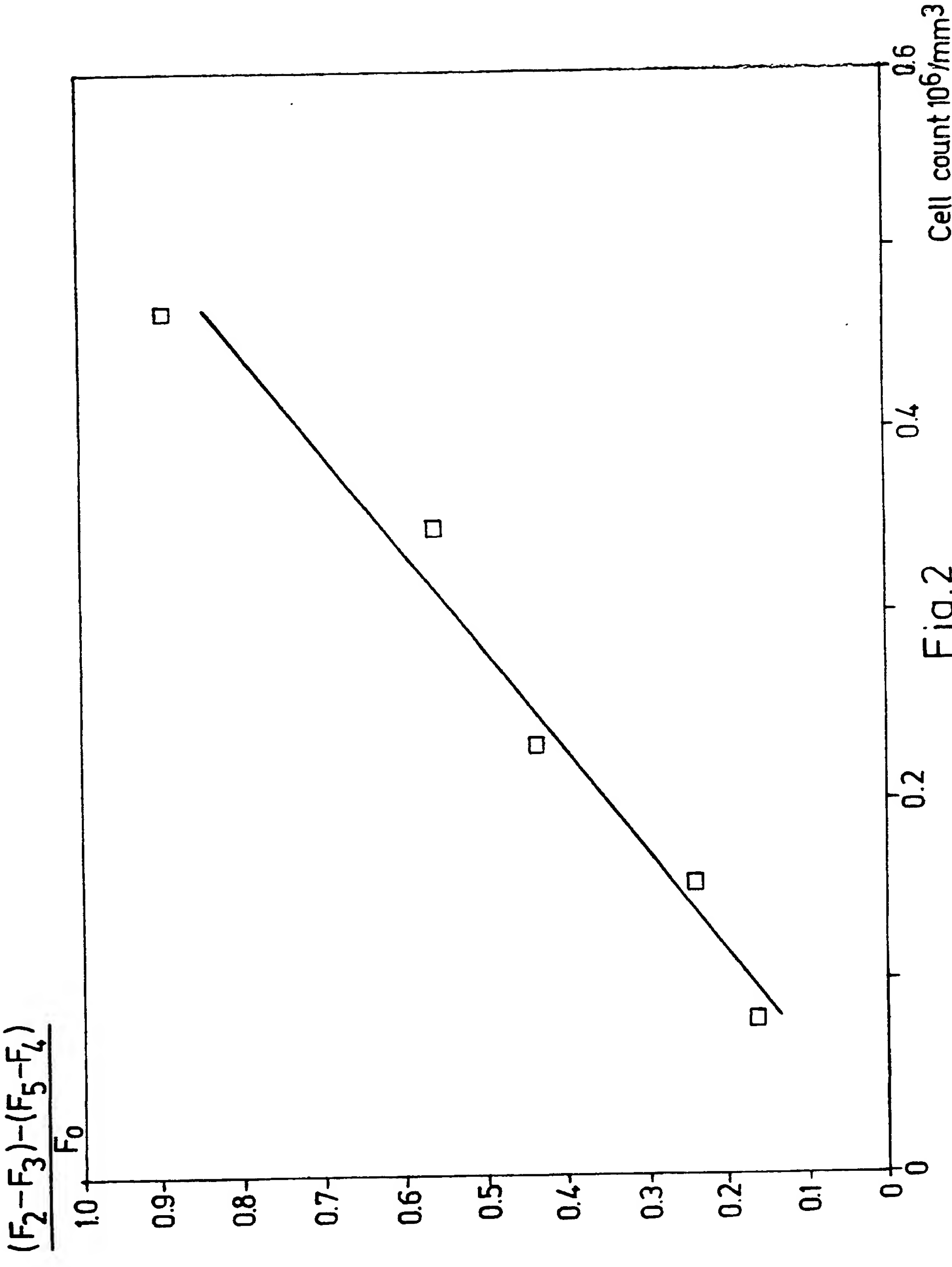
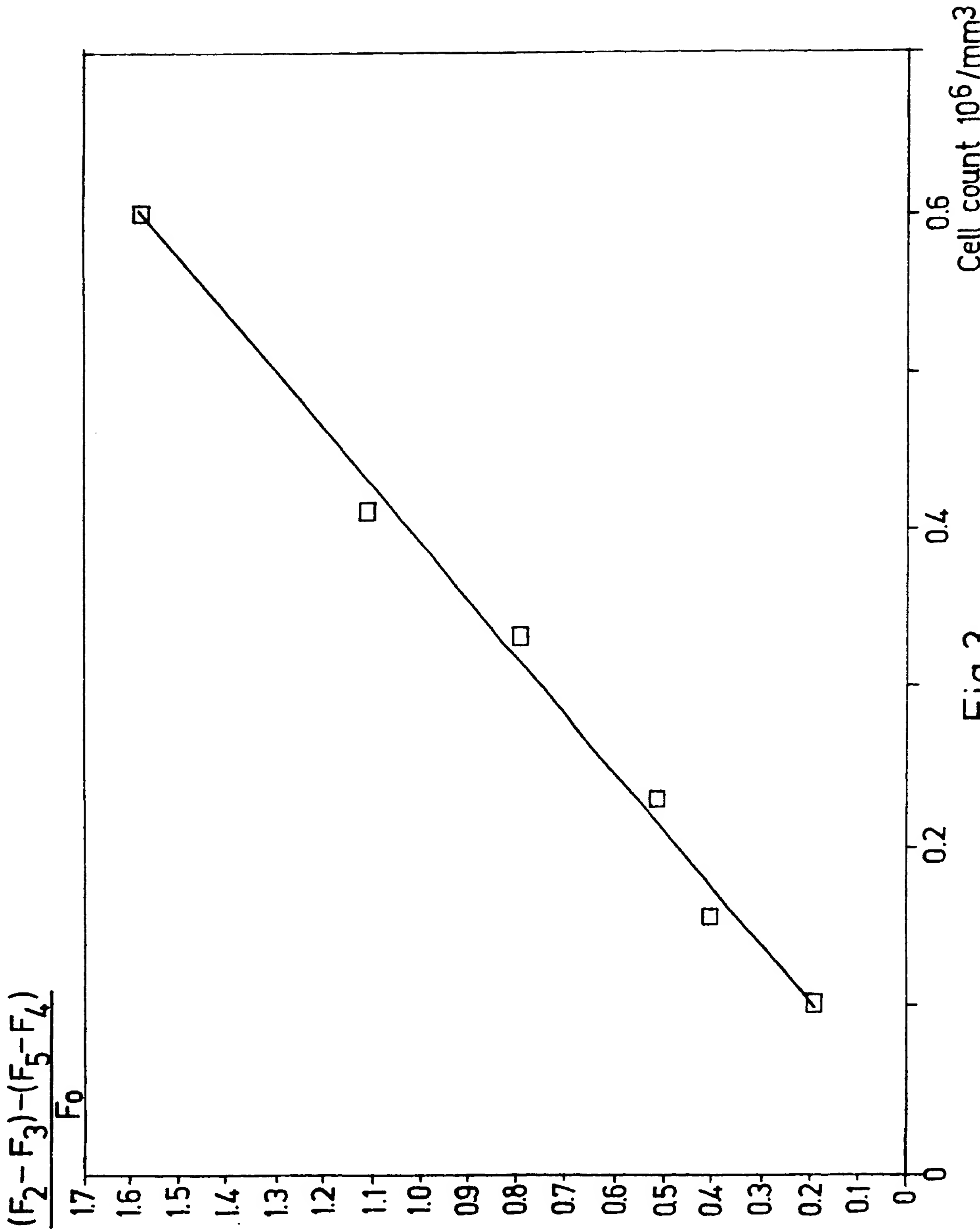

$\frac{(F_2-F_3)-(F_5-F_4)}{F_0}$
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
Cell count $10^6/mm^3$

Fig.2



$\frac{(F_2-F_3)-(F_5-F_4)}{F_0}$
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
Cell count $10^6/mm^3$

Fig.3

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PROCESS AND EQUIPMENT FOR THE RAPID DETERMINATION OF THE SPERMIUM CELL COUNT AND/OR LIVING SPERMIUM COUNT

This invention relates to a process and equipment for the rapid determination of the spermium cell count and/or the ratio of living spermia in sperm samples.

Research work connected with spermia cannot be separated from economical viewpoints, production interests of artificial insemination and practical animal husbandry as well as the demand to increase production. Thus, a number of research programs have been directed to an objective qualification of sperm, more particularly to the development of processes by means of which the biological utility of the sperm could be predicted in practice.

In addition to the traditional staining techniques, several methods are today available for investigators that make it possible to observe very fine alternations of the qualitative, morphological and physiological characteristics of the sperm, such as electron microscopy /¯J. M. Bedford: Am. J. Anat. 123, 329 (1968); Saacke: J. Amin. Sci. 115, 143 (1964)7, enzymological analysis /¯Acta Vet. Scand. 17, 83 (1976)7 and methods based on laser techniques /¯Magyar Állatorvosok Lapja 38, 38 (1983); Biophys. J. 31, 147 (1983)7.

In sperm, cells in various physiological conditions are present, and the ratio of these subpopulations plays a

decisive role in insemination.

Nowadays, microscopic investigation is still the most widely used routine examination method for estimating the concentration, mobility and thus the ratio of living spermia; by using an appropriate enlargemant and observing the spermia within the sight field, the investigator subjectively estimates the biological utility of the sperm tested on the basis of movement of the spermia. This method is inaccurate and shows an error of about 25% /‾Techn. Conf. Art. Ins. Reprod. Proc. p. 3 (1973)̲7.

This qualification can be supplemented with various staining techniques. Namely, the living subpopulation can be distinguished from the dead one by using so-called vital dyeing since the dye is not taken up by the originally living cells. In this case, the inaccuracy of this method arises from the fact - that a population containing several millions of cells is qualified on the basis of examination of a few hundred cells. Furthermore, this method is time-consuming and cannot be fitted to production technology.

An other known method is based on the observation that a velocity-dependent frequency-modulated component is contained in the light scattered by the head section of spermia when the sperm sample is illuminated by the monochromatic light of a He-Ne laser. The velocity distribution of the spermia can be concluded by Fourier transformation of frequency spectrum of the Doppler signal. This method is also useful for measuring the spermium concentration (LAZYMOT device of BIG Biotechnik GmbH., Mönchengladbach, German Federal

Republic, used for investigation of the mobility).

On the one hand, the known methods are not suitable to assure an accurate and reproducible result and, on other hand, they are too complicated and expensive for the wide use in industrial processes for working-up of sperm.

The aim of the present invention is to provide a process and equipment for carrying out a simple and relatively inexpensive qualification of sperm with an accuracy satisfying production requirements.

The invention is based on the recognition that the fluorescence characteristics of propidium iodide such as the spectral distribution and the quantum efficiency of fluorescence of the emitted light are altered by the interaction with and binding of the dyestuff to nucleic acids. The binding of the dyestuff molecules appears as a significant increase in the fluorescence level which is characteristic of the free dyestuff.

Sperm samples are qualified on the basis that, in contrast with the intact cells, propidium iodide is very quickly taken up by the dead cells having a membrane with enhanced permeability whereby an increase in the intensity is observed as compared to that of the free dyestuff. This is a distinctly rapid process whereby an even higher fluorescence intensity is obtained which is proportional to the total spermium count.

Thus, the present invention relates to a process for the rapid determination of the spermium cell count of sperm samples and/or the ratio of the living spermia, which

comprises dissolving the fluorescent dyestuff propidium iodide in a buffer and measuring the intensity (F_0), then adding the sperm sample and, after measurement of the intensity (F_1) and addition of a cytoplasm membrane--permeabilizing agent, determining the intensity (F_2), then adding a buffer and a permeabilizing agent to the buffer and measuring the intensity (F_3) and subsequently the intensity (F_4) of the pure buffer and finally the intensity (F_5) of the sperm mixture added to the buffer, and calculating the cell concentration by using the formula:

$$\text{cell concentration} = \alpha \cdot \frac{(F_2-F_3) - (F_5-F_4)}{F_0} \text{ million/mm}^3.$$

wherein α is the multiplication product from the slope of the calibration curve with the predilution ratio; and calculating the living cell ratio by using the formula:

$$\text{living cell \%} = 100 - \frac{(F_1-F_0) - (F_5-F_4)}{(F_2-F_3) - (F_5-F_4)} \cdot 100.$$

As a cytoplasm membrane-permeabilizing agent, saponin, digitonin or nystatin may preferably be added to the stained cell sample whereby the membrane of each cell becomes permeable.

Further on, the invention relates also to an equipment for carrying out the above process, which comprises: a cuvet-holder unit (1); a light source (5) connected to it; an input-optic including an optical filter (4) and two

lenses (3) which, after choosing the appropriate spectral range, project the light of the light source to the sample placed on the thermostatic cuvet-holder (2); an output--optic which is perpendicular to the axis of the input--optic and includes an optical filter (7) and two lenses (6) which, through the light detector (8) joining to the cuvet--holder unit, projects the emission of the sample (11) placed in the cuvet-holder; a high-voltage supply unit (9) for the light detector; and detecting electronics (10) receiving the signals of the light detector.

A scheme of the equipment according to the invention is shown in Figure 1.

The equipment functions as follows: The device is started up by switching on the mains switch and adjusting a high voltage value on the high-voltage supply unit (9) feeding the light detector (8). The high-voltage value assures the display of an emission intensity which is well measurable with the selected amplifying field by the detecting electronics (10) receiving the signals of the light detector.

For measuring the emission intensity of the samples used for the determinations, the sample placed in the cuvet to be measured is put into the cuvet-holder (2), whereafter the emission intensity is automatically displayed by the detecting electronics (10) of the equipment.

For carrying out a complete series of measurements, i.e. for determining the living cell ratio of a sperm sample to be qualified, the emission intensity of six samples

prepared in different ways has to be measured. Thereafter, the evaluation is made by determining the living cell ratio as well as the absolute cell concentration of the sample to be qualified.

The extent of amplification as well as of the filtration diminishing the electronic background noise may be varied according to several grades. Suitably, the value of the high voltage switched on the photoelectron-multiplier used as detector is let to appear on a recorder.

The main advantages of the process and equipment of the invention may be summarized as follows.

a) They are more simple and inexpensive than any of the prior art.

b) Accurate and reproducible results are rapidly obtained which can thus be inserted into the technology of working up the sperm.

c) They are useful to determine the accurate ratio of the living cells to the dead ones.

d) An objective establishment of the resistance of an optional ejaculate and conclusively the prediction of the biological value of the sperm tested are made possible by the serial examinations of sperm samples subjected to a loading test.

e) The measurement of the absolute spermium concentration also becomes possible.

f) The process is more sensitive than those of the prior art.

g) On binding of the propidium iodide dyestuff, the

fluorescence significantly increases, resulting in a higher resolving power.

The process of the invention is further illustrated in detail by the following non-limiting Example.

A calibration curve of boar sperm is shown in Figure 2 and that of bull sperm in Figure 3.

$\frac{25\,mg}{l} \times \frac{1\,mM}{668\,mg} = 0.037\,mM = 37\,\mu M$

Example

PI = 668 mw

The ratio of the living cells is determined as follows.

1. The fluorescence intensity (F_0) of propidium iodide (PI) dissolved in a buffer is measured on 1600 μl of a dyestuff solution having 25 mg/litre concentration of PI and serving also as standard.

2. The sperm sample to be tested pre-diluted with 40 μl of the buffer is added and the intensity (F_1) is measured.

3. As a permeabilizing agent 40 μl of an ethanolic solution containing 5 mg/ml of digitonin are added to the cell sample and the intensity (F_2) is measured.

4. Instead of the sperm sample, 40 μl of PBS (phosphate buffer saline) and then the permeabilizing agent are added to the dyestuff solution, and the fluorescence intensity (F_3) of the thus-prepared solution is determined (1600 μl of PI solution + 40 μl of buffer + 40 μl of ethanolic digitonin solution).

5. The intensity (F_4) of 1600 μl of the buffer solution is measured.

6. The intensity (F_5) is determined after adding 40 μl

of the pre-diluted sperm to the buffer.

The ratio of intact cells is calculated from the intensities measured by using the formula:

$$\text{Living cells \%} = 100 - \frac{(F_1-F_0) - (F_5-F_4)}{(F_2-F_3) - (F_5-F_4)} \cdot 100$$

The cell count is determined as follows: The cell count contained in the sample can simultaneously be determined from the intensities measured above within the serial measurements for determining the living cell ratio. Namely, the difference between the measured intensities F_2 and F_3, i.e. the value of (F_2-F_3), originates from the total permeabilized cells and therefore it is proportional to the cell count of the sample. Within the range $1x10^5$ to $1.5x10^6$ cell/ml the value of (F_2-F_3) shows a linear correlation to the cell concentration. Thus, within this range, by means of calibration the cell count of the sample can be obtained from the value of (F_2-F_3) measured in the course of the process. The non-specific intensity enhancement (F_5-F_4) resulting from the spermia has to be considered in this case, too.

The calculation is carried out as follows:

$$\text{Spermium concentration in million/mm}^3 = \alpha \cdot \frac{(F_2-F_3) - (F_5-F_4)}{F_0}$$

wherein α has the above value.

37μM / 0.03ml = x / ml =

The calibration curve can be prepared by using a flow-cytometer and a cell sample labelled with PI as follows.

The cell suspension to be tested is diluted to a concentration of 0.5×10^6 to 2×10^6 cell/ml by using PBS buffer. Under cooling with ice, 1 ml of the diluted suspension is treated with 1 ml of 1% NP-40 solution (Nonidet P-40, SIGMA, Deisenhofen, German Federal Republic). Under the effect of this treatment, all cells perish and their membranes become permeable for PI.

2 ml sample

After this treatment, the sample is stained by adding PI dissolved in PBS buffer up to a final concentration of 30 μl/ml of the dyestuff.

0.030mls stock 37μM added to 1ml

The stained sample is analyzed in a ... ,
e.g. in a Dickins ... ate
of 1500 cell/sec. ... on
laser at 488 nm w ... d
by inserting a sup ... le
in the range above

$$\frac{37\,\mu\text{moles}}{1000\,\text{mls}} \times 0.030\,\text{mls} = \frac{0.0011\,\mu\text{moles}}{1\,\text{ml}}$$

In the cour
through the system
of registering the
PI is uniformly tak
effect of the treat
absolute cell count ... e
amount of sample co ... l
the dilution factor
sample is obtained w
intensity quotient

1.11 μMolar

$$\frac{(F_2-F_3) - (F_5-F_4)}{F_0}$$

measured on the same sample in a spectrofluorimeter. On preparing a dilution series from the sample and using a series of parallel cytometric and spectrofluorimetric measurements, the absolute cell counts belonging to the intensity quotient measured in the spectrofluorimeter are determined in various concentration ranges, the accuracy of which is assured by examinations repeated several times at the same points of measurements. Since the value of the quotients of the fluorescence intensities is in a linear correlation to the cell count, a regression line of the measurements for any sperm species can be plotted, on the basis of which the cell count belonging to a defined intensity quotient can be determined.

The multiplication product from the slope of the regression line with the dilution of the sperm sample tested gives the coefficient α which, on multiplying with the fluorescence intensity quotient measured, affords the original cell concentration of the sample.

The callibration curve may also be constructed by haemocytometric counting.

The calculations may preferably be performed on a computer.

Claims

1. A process for the rapid determination of the spermium count of sperm samples and/or the ratio of the living spermia, which comprises dissolving the fluorescent dyestuff propidium iodide in a buffer and measuring the intensity (F_0), then adding the sperm sample and, after measurement of the intensity (F_1) and addition of a cytoplasm membrane--permeabilizing agent, determining the intensity (F_2), then adding a buffer and a permeabilizing agent to the buffer and measuring the intensity (F_3) and subsequently the intensity (F_4) of the pure buffer and finally the intensity (F_5) of the sperm mixture added to the buffer, and calculating the cell concentration by using the formula:

$$\text{cell concentration} = \alpha \cdot \frac{(F_2-F_3) - (F_5-F_4)}{F_0} \text{ million/mm}^3$$

wherein α is the multiplication product from the slope of the calibration curve with the predilution ratio; and calculating the living cell ratio by using the formula:

$$\text{living cell \%} = 100 - \frac{(F_1-F_0) - (F_5-F_4)}{(F_2-F_3) - (F_5-F_4)} \cdot 100.$$

2. A process as claimed in claim 1, which comprises using propidium iodide in a concentration of from 12.5 mg/litre to 25 mg/litre.

3. A process as claimed in claim 1, which comprises using saponin, digitonin or nystatin as cytoplasm membrane-permeabilizing agent.

4. An equipment for carrying out the process as claimed in claim 1, which comprises a cuvet-holder unit (1); a light source (5) connected to it; an input-optic including an optical filter (4) and two lenses (3) which, after choosing the appropriate spectral range, project the light of the light source to the sample placed on the thermo-statable cuvet-holder (2); an output-optic which is perpendicular to the axis of the input-optic and includes an optical filter (7) and two lenses (6) which, through the light detector (8) joining to the cuvet-holder unit, projects the emission of the sample (11) placed in the cuvet-holder; a high - voltage supply unit (9) for the light detector; and detecting electronics (10) receiving the signals of the light detector.

5. Equipment as claimed in claim 4 which includes means for computing the percentage of living cells and/or the cell concentration in a semen sample by performing the calculations set out on claim 1.

6. A process as claimed in claim 1, substantially as hereinbefore described in the Example.

7. Equipment substantially as hereinbefore described in connection with and as illustrated in Figure 1 of the accompanying drawings.

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